Analysis of the Serologic Determinant Groups of the Salmonella E-Group O-Antigens*

T. UCHIDA,† P. W. ROBBINS, AND S. E. LURIA

From the Department of Biology, Massachusetts Institute of Technology, Cambridge

Received February 4, 1963

In a previous paper (Robbins and Uchida, 1962a) the chemical structures of the O-antigen of *Salmonella anatum* and of lysogenic strains derived from this organism were described. It was found that lysogenization by certain bacteriophages leads to simple, well-defined changes in the chemical structure of the O-antigen. The nonlysogenic organism was found to have a polysaccharide O-antigen that contained the primary sequence 6-*O*-α-D-galactopyranosyl-D-mannosyl-L-rhamnose. After infection with ϵ^{15} the lysogenic organism contained a polysaccharide with the sequence 6-*O*-β-D-galactopyranosyl-D-mannosyl-L-rhamnose. Cells that carry both ϵ^{15} and ϵ^{34} have an O-antigen with the sequence *O*-α-D-glucopyranosyl-(1 → 4)-*O*-β-D-galactopyranosyl-D-mannosyl-L-rhamnose. In the present paper these studies have been extended by detailed serologic analysis. This analysis shows that there is a direct correlation between the serologic and chemical changes detected after lysogenization. It also has led to the discovery of an *O*-acetyl group as part of determinant 10 and has furnished strong evidence that nonterminal regions of a polysaccharide structure may serve as serologic determinant groups.

The chemical structures of the antigenic polysaccharides of group E Salmonella that are controlled by bacteriophages have been reported (Robbins and Uchida, 1962a). Organisms in group E_1 carry O-antigen 3, 10 and are susceptible to phage ϵ^{15}, which changes the O-antigen to 3, 15—that of group E_2. The ϵ^{15}-lysogenic organisms thus obtained become susceptible to another phage, ϵ^{34}, which converts antigen 3, 15 to (3), (15), 34, that is, to the antigen of group E_4. Chemical analyses revealed that the structural units of antigens 3, 10 and 3, 15 are, respectively, 6 - *O* - α - D - galactopyranosyl - D - mannosyl - L-rhamnose and 6-*O*-β-D-galactopyranosyl-D-mannosyl-L-rhamnose. It was also found that a 4-*O*-α-D-glucopyranosyl-D-galactose residue is the essential part of determinant 34 in antigen (3), (15), 34. This paper will report the details of serologic analysis of these antigenic determinants. In addition, evidence will be presented that attributes the specificity of determinant 3 to a mannosyl-rhamnose residue and that identifies the structural unit of antigen 10 as an acetyl-galactosyl-mannosyl-rhamnose.

MATERIALS AND METHODS

Bacterial Strains.—*S. anatum* strain 1 (A_1), its lysogenic derivatives $A_1(\epsilon^{15})$, $A_1(\epsilon^{34})$, and $A_1(\epsilon^{15}, \epsilon^{34})$ were used as group E organisms. Strain A_1 was chosen instead of strain A (*S. anatum* 293: Uetake *et al.*, 1958) because of the greater stability of its S form. Other strains described in the text are International standards.

Antigens.—Lipopolysaccharides extracted by the phenol-water method (Westphal *et al.*, 1952) were used for serologic reactions. For the lipopolysaccharide of group E_1 Salmonella, the organism $A_1(\epsilon^{34})$ carrying antigen 3, 10 was used because of the ease of extraction (Robbins and Uchida, 1962a).

Lipopolysaccharides from $A_1(\epsilon^{34})$, $A_1(\epsilon^{15})$ and $A_1(\epsilon^{15}\epsilon^{34})$ were further treated with cetyl trimethyl ammonium bromide to remove any contaminating nucleic acid. The preparations contained no nucleic acid detectable by absorption spectrum.

* Supported by Grant-8808 from the National Science Foundation and grants E-3038 and A-3772 from the National Institutes of Health.

† Present address: Sapporo Medical College, Sapporo, Japan. The experiments presented in this paper were carried out while the author was on leave of absence from Sapporo Medical College.

Oligosaccharides.—Partial hydrolysis of lipopolysaccharides was carried out in 1 N H_2SO_4 for 20 minutes at 100°. After neutralization with $Ba(OH)_2$ and concentration in a vacuum, the solutions were applied to a charcoal-Celite column and eluted with a linear gradient prepared by allowing 30% ethanol to run into a mixing chamber containing water. The separated fractions were concentrated and purified further by paper chromatography on Whatman 1 with the solvent system 1-butanol, pyridine, water (6:4:3). The various carbohydrates were eluted and their identity was checked by paper chromatography and paper electrophoresis. When necessary, the products were further fractionated by electrophoresis on Whatman 1 with 0.05 N sodium borate. Carbohydrate concentrations were determined by the phenol–sulfuric acid method (Smith and Montgomery, 1956). Other methods are described by Robbins and Uchida (1962a).

Antisera.—For preparation of immunogens (Kauffmann, 1954) all organisms were washed twice with physiologic saline, resuspended, heated at 100° for 2.5 hours, and then washed once with saline. Because of the toxicity of these heated organisms, treatment with chrome alum (potassium chrome sulfate) (Ando and Nakamura, 1951) was used as follows. Heated and washed cells were suspended in sterile saline containing 0.02% merthiolate to give a suspension of 100 mg wet cells per ml. Cell suspensions were mixed with 0.1 volume of 1% sterile chrome alum (heated at 100° for 30 minutes). After the mixture was kept at room temperature for 3 days, the cells were resuspended in two volumes of sterile saline containing 0.01% merthiolate. Rabbits were injected intravenously every 4 days with 0.5, 1.0, 1.5, and 2.0 ml of cell suspensions. In most cases, 2 to 4 weeks after the last injection the rabbits received a booster injection of 1.0 ml of cell suspension and 1 week later were bled. Antisera from each rabbit were heated separately at 56° for 30 minutes and stored frozen. *S. newington* C_2 and *S. niloese* 1236 were used for preparation of anti-3, 15 serum and anti-1, 3, 19 serum, respectively. Monofactor sera were prepared as follows. Undiluted whole serum was mixed with heated and washed organisms at 50° for 2 hours and kept at 3° for 2 days; 500 mg of wet cells was used per ml of serum. Anti-34 serum was prepared from anti-$A_1(\epsilon^{15}\epsilon^{34})$ serum by absorbing with $A_1(\epsilon^{15})$ or with both $A_1(\epsilon^{15})$ and *S. typhi* 0901. Anti-15 serum was prepared from anti-*S. newington* serum by absorbing with

A_1, and anti-10 serum from anti-A_1 serum by absorbing with $A_1(\epsilon^{15})$, *S. senftenberg*, and *S. minnesota* (Kauffmann, 1961). After absorption, the supernatant fluid was kept at 3° for 1 week to remove any precipitate. Monofactor sera were checked by agglutination, precipitation, and complement-fixation reactions and were stored frozen.

Test Immune Systems.—For the 34, 15, and 10 immune systems each monofactor serum and corresponding lipopolysaccharide were used. For the 3 immune system the following three cross reactions were employed: lipopolysaccharide, 3, 15 with anti-3, 10 serum, lipopolysaccharides 3, 10 and 3, 15 with anti-1, 3, 19 serum.

Inhibition Analysis.—The serologic activity of oligosaccharides obtained from partial acid hydrolyzates of lipopolysaccharide was tested by inhibition analysis. The micro-complement fixation method (Wasserman and Levine, 1961) was used throughout. Isotonic Veronal buffer containing 5×10^{-4} M $MgCl_2$, 1.5×10^{-4} M $CaCl_2$, and 0.1% bovine serum albumin was used as a diluent. Sheep red blood cells were obtained from Probio, Inc., and antisheep hemolysin from Carworth Laboratories, Inc. Since complement (Cappel Laboratories) contained a substance serologically active against lipopolysaccharides (3), (15), 34 and 3, 15, the complement was absorbed by mixing with an equivalent amount of lipopolysaccharide (3), (15), 34 at 0° overnight and centrifuging at 30,000 rpm for 1 hour at 0°. The clear supernatant fluid was stored at −20°. Nonspecific reactions with the lipopolysaccharides disappeared and no significant decrease in complement activity was observed.

Complement diluted 1:250 was used. For each immune system, quantities of antigen and antibody were chosen to give the maximum peak fixation. Reagents were mixed at 0°; the final volume was 3.0 ml. After incubation at 3° for 18 hours, 0.5 ml of sensitized red cells diluted 1:10 (2.5×10^7 cells) was added and the mixture was kept at 37° for 50 to 60 minutes. Sensitization was done to give 70 to 80% hemolysis in controls. Hemolysis was measured in a Zeiss spectrophotometer at 413 mμ. During dilution of oligosaccharide preparations special precautions were taken to eliminate changes in the ionic strength of the reaction mixture. The reagents of all systems and oligosaccharides used caused no anticomplementary or lytic affects. The per cent of complement fixation was calculated as follows:

$$\frac{\text{OD of antibody control} - \text{OD of antigen-antibody reaction mixture}}{\text{OD of antibody control} - \text{OD of blank}} \times 100$$

The per cent inhibition was calculated from the formula:

$$\frac{\text{OD in the presence of oligosaccharide} - \text{OD of immune system}}{\text{OD of antibody control} - \text{OD of immune system}} \times 100$$

Complement fixation curves were drawn by plotting the per cent fixation on a linear scale as a function of quantity of lipopolysaccharide on a logarithmic scale. In the figures, inhibition data are presented by plotting the per cent inhibition on a linear scale as a function of the quantity of inhibiting material on a logarithmic scale.

Enzyme Treatment.—To remove a nonreducing terminal galactosyl group from a β-galactosyl-mannosyl-rhamnosyl trisaccharide the oligosaccharide preparation was treated at 25° with β-galactosidase prepared from *E. coli* (Robbins and Uchida, 1962a). The digested materials were subjected to paper chromatography and oligosaccharides were eluted from the appropriate areas.

Determination of O-Acyl Group.—Acetyl content of lipopolysaccharides and of acetylated oligosaccharides was measured essentially by the method of Hestrin (1949). One ml of sample containing 1 to 3 mg of lipopolysaccharide was mixed with 2.0 ml of a freshly prepared mixture of equal volumes of 2 M hydroxylamine hydrochloride and 3.5 N NaOH. Two minutes after mixing, 1.0 ml of 3 N HCl containing 25% trichloroacetic acid and then 1.0 ml of 0.37 M ferric chloride in 0.1 N HCl were added. The color was read immediately in a Klett Summerson colorimeter with filter 45. Controls were set by adding the acid solution before the hydroxylamine solution. All readings were corrected by subtracting those of controls. The absorption spectrum was also checked against that of the ferric–acetyl hydroxamate complex. As a standard solution succinic hydroxamate was used (Lipmann and Tuttle, 1945). Chromogenic activity of the succinohydroxamic acid ferric complex was checked by means of acetylcholine (Sigma). The acetyl content of acetylcholine was measured as follows: A solution containing 1.0 mg of acetylcholine in 5.5 ml of H_2O was diluted 1:10 with water and treated with an equal volume of standard 0.2 N NaOH. After 30 minutes, free NaOH was back-titrated with 0.1 N HCl to a bromothymol-blue neutral point. The acetyl content was 85% of that expected for pure acetylcholine. The acetylcholine was then used for the preparation of a standardized acetylhydroxamic acid solution. The chromogenic activity of the acetylhydroxamate was found to be 3% greater than that of the succinic hydroxamate standard. Acetyl groups were detected qualitatively by comparing on paper chromatograms the hydroxamate derived from the lipopolysaccharide with a known acetylhydroxamate (Fink and Fink, 1949).

Acetylation of Oligosaccharides.—A solution containing 1 to 10 μmoles of oligosaccharide was treated with acetic anhydride at room temperature. Samples were neutralized with 1 N NaOH to bring the *p*H to 7.0. Acetylated samples were diluted with water for the measurement of acetyl content and for the inhibition analysis.

Results

Determinant 34.—From the basic formulation of the E-group antigens presented in Figure 1 (Robbins and Uchida, 1962a), it is expected that the 34 determinant group will center around a terminal α-1,4-D-glucosyl residue. Inhibition studies clearly show that α-1,4-D-glucosyl-D-galactose is the essential component of the 34 determinant (Fig. 2). The trisaccharide α-1,4-D-glucosyl-β-1,6-D-galactosyl-mannose also inhibited the 34 immune system. The trisaccharide β-1,6-D-galactosyl-mannosyl-rhamnose obtained from antigen (3), (15), 34 inhibited this system weakly, but no inhibition was observed when the same trisaccharide was prepared from antigen 3, 15; this suggests that the weak inhibition is caused by contamination with the α-glucosyl-galactosyl-mannose trisaccharide.

Since antisera for group E_3 Salmonella organisms contain antibody cross-reacting with *S. typhi* antigen 9, 12 (Kauffmann, 1942, 1961), the anti-34 serum was further absorbed with *S. typhi* 0901 and subjected to the inhibition analysis. The disaccharide glucosyl-galactose inhibited the reaction of this absorbed anti-34 serum to the same extent as that shown in Figure 2. This shows that the inhibition results are relevant to the 34 system and are not merely the reflection of the

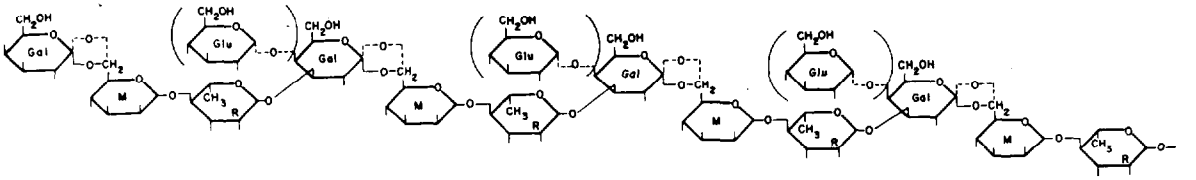

FIG. 1.—Composite structure of the three E-group antigenic side chains. Antigen 3, 10 is represented by the solid lines showing α-D-galactosyl residues (└O—). In antigen 3, 15 the galactosyl residues are present as the β-anomers and are shown by dotted lines (┌O--). Antigen (3), (15), 34 contains the β-galactosyl residues and in addition has the α-D-glucosyl residues shown in parentheses.

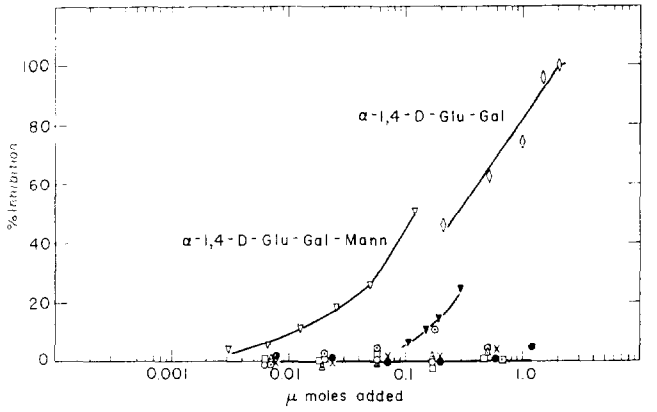

FIG. 2.—Inhibition of system 34 (lipopolysaccharide (3), (15), 34 and anti-34 serum) by oligosaccharides. The experiments were carried out as described in the text. The symbols used in this and following figures for oligosaccharides are as follows: ○, D-mannosyl-L-rhamnose of lipopolysaccharide 3, 10; □, D-mannosyl-L-rhamnose of lipopolysaccharide 3, 15; ⊙, α-1,6-D-galactosyl-D-mannose of lipopolysaccharide 3, 10; △, β-1,6-D-galactosyl-D-mannose of lipopolysaccharide 3, 15; ⊡, β-1,6-D-galactosyl-D-mannose of lipopolysaccharide (3), (15), 34; ×, α-1,6-D-galactosyl-1, 4 or 1, 5-D-mannosyl-L-rhamnose of lipopolysaccharide 3, 10; ●, β-1,6-D-galactosyl-1, 4 or 1, 5-D-mannosyl-L-rhamnose of lipopolysaccharide 3, 15; ▼, β-1, 6-D-galactosyl-1, 4 or 1, 5-D-mannosyl-L-rhamnose of lipopolysaccharide (3), (15), 34; ◊, α-1, 4-D-glucosyl-D-galactose of lipopolysaccharide (3),(15),34; ▽, α-1,4-D-glucosyl-β-1,6-D-galactosyl-D-mannose of lipopolysaccharide (3), (15), 34.

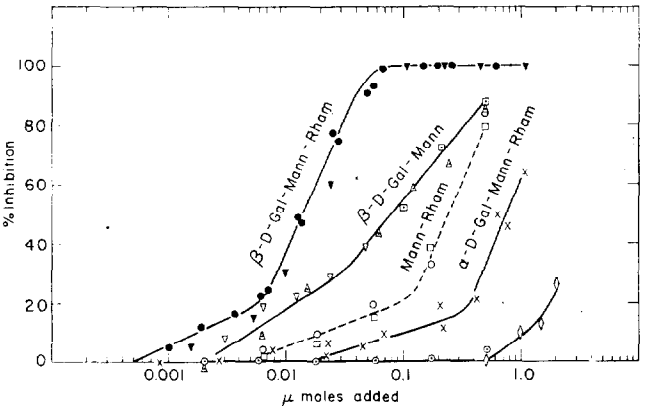

FIG. 3.—Inhibition of system 15 (lipopolysaccharide 3, 15 and anti-15 serum) by oligosaccharides. Symbols explained in Fig. 2.

cross-reacting system. There is evidence in the literature that an α-1,4-D-glucosyl-galactosyl structure is responsible for the determinant of antigen 12 (Staub, 1960; Tinelli and Staub, 1960).

In tests using the precipitin reaction, glycogen reacted with the anti-34 serum; 15% of the antibody protein precipitable by lipopolysaccharide (3), (15), 34 was precipitated by glycogen. This observation further supports the proposed structure for determinant 34.

Determinant 15.—As shown in Figure 3, β-1,6-D-galactosyl-mannosyl-rhamnose trisaccharide obtained from either antigen 3, 15 or antigen (3), (15), 34 strongly inhibited the 15 immune system. Disaccharide β-1,6-D-galactosyl-mannose also inhibited the system, although to a lesser extent. Some inhibition was observed with D-mannosyl-rhamnose. No inhibition was observed with α-1,6-D-galactosyl-mannose from antigen 3, 10. It is evident from the comparison of the inhibiting powers of β-1,6-D-galactosyl-mannose and D-mannosyl-rhamnose that the 15 determinant centers about the β-1,6-D-galactosyl residue. Mannosyl-rhamnose disaccharide obtained from both antigens 3, 10 and 3, 15 inhibited the 15 system to the same extent, an indication of the identity of this disaccharide in both structures. Trisaccharide α-1,6-D-galactosyl-mannosyl-rhamnose inhibited the reaction weakly, probably because of the incorrect configuration of the galactosyl residue. From these results it is concluded that β-1,6-D-galactosyl-mannosyl-rhamnose is largely responsible for the 15 determinant site. It is interesting to note (Fig. 3) that α-1,4-D-glucosyl-β-1,6-D-galactosyl-mannose inhibited the 15 system to the same extent as the β-1,6-D-galactosyl-mannose disaccharide. Although there is a possibility that the former would be contaminated by some β-1,6-D-galactosyl-mannosyl-rhamnose trisaccharide, the slope of the inhibition curve is different from that of this trisaccharide. This finding suggests that the internal structure of galactosyl-mannose is able to supply the binding power without any interference from the terminal glucosyl residue. This possibility will be discussed later.

Determinant 3.—Inhibition of the 3 immune system was carried out by use of the cross reactions of lipopolysaccharide 3, 15 and anti-3, 10 serum and of lipopolysaccharide 3, 10 and 3, 15 with anti-1, 3, 19 serum. The mannosyl-rhamnose disaccharide and the galactosyl-mannosyl-rhamnose trisaccharides, as shown in Figures 4, 5, and 6, inhibited all three immune reactions. It is

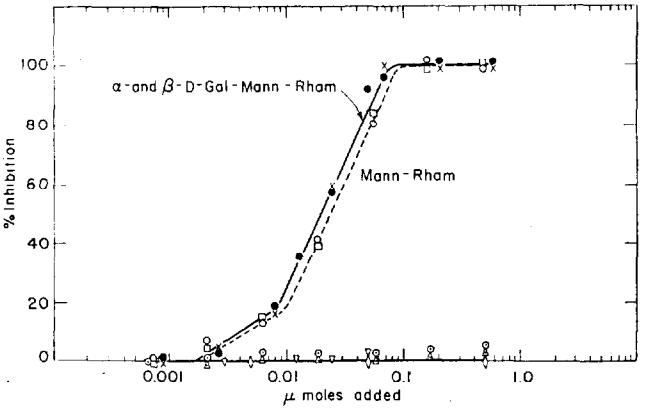

FIG. 4.—Inhibition of system 3 (lipopolysaccharide, 3, 15 and anti 3, 10 serum) by oligosaccharides. Symbols explained in Fig. 2.

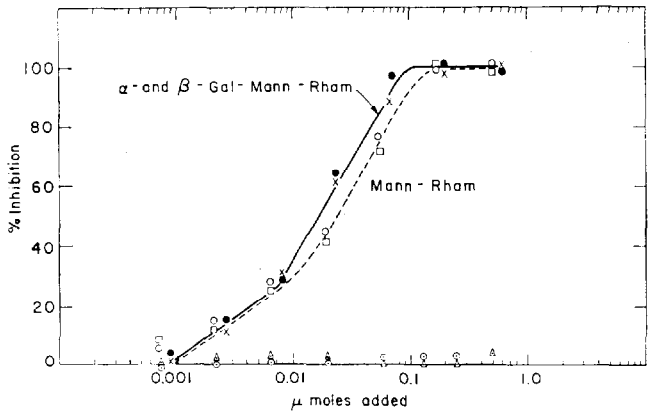

FIG. 5.—Inhibition of system 3 (lipopolysaccharide 3,10 and anti 1, 3, 19, serum) by oligosaccharides. Symbols explained in Fig. 2.

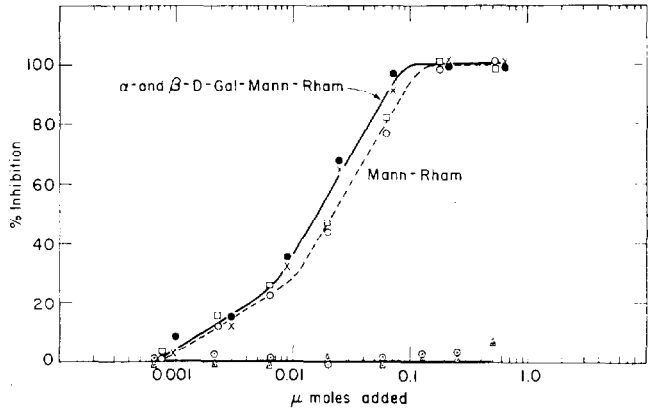

FIG. 6.—Inhibition of system 3 (lipopolysaccharide 3, 15 and anti 1, 3, 19 serum) by oligosaccharides. Symbols explained in Fig. 2.

surprising that the extent of inhibition by all these oligosaccharides was very similar and independent of their source. The results indicate that the 3 determinant centers about the mannosyl-rhamnose grouping. Similar results were obtained in tests with lipopolysaccharide 3, 10 and anti-3, 15 serum, but because of anticomplementary activity of the antiserum the results could not be compared with the others.

The remarkable finding is that both α- and β-galactosyl-mannosyl-rhamnose trisaccharides inhibited the reactions and gave essentially identical activities very close to that shown by the mannosyl-rhamnose disaccharides. According to Kabat (1956, 1958, 1961) nonreducing terminal groupings are responsible for the determinants in polysaccharide antigens. It seemed possible that a contaminant carrying a mannosyl residue as a nonreducing terminal moiety might be responsible for the inhibition. To test this possibility, the β-1,6-D-galactosyl-mannosyl-rhamnose was hydrolyzed with β-galactosidase and residual trisaccharide was reisolated. If a trisaccharide having a mannosyl end-group had been responsible for the inhibiting ability of the preparation, one would expect that the enzyme treatment would cause an enrichment of the contaminant, resulting in the increase in specific inhibiting power of the residual trisaccharide. That this is not the case is shown in Table I. There is no significant decrease in the concentration causing 50% inhibition even though more than 90% of the β-galactosyl-mannosyl-rhamnose preparation was hydrolyzed.

These findings indicate that the nonreducing terminal group of the β-galactosyl-mannosyl-rhamnose trisaccharide does not interfere with the binding between the

TABLE I

INHIBITION OF SYSTEMS 3 AND 15 BY ENZYME-TREATED TRISACCHARIDE

The values in the table are the amounts of trisaccharide in μmoles that give 50% inhibition.

Immune System	Untreated Trisaccharide	Enzyme-Treated and Reisolated Trisaccharide	
		Degree of Hydrolysis[a]	
		60%	95%
System 3	0.0196	0.0159	
	0.0172		0.0132
System 15	0.0125	0.0153	

[a] Total recovery of carbohydrates eluted from the paper was 90% of starting materials. The degree of hydrolysis was calculated as follows:

$$\left(1 - \frac{\text{recovered trisaccharide}}{\text{total recovered carbohydrate}}\right) \times 100$$

internal mannosyl-rhamnose segment and the specific antibody-combining site. The terminal residue even of the α-galactosyl-mannosyl-rhamnose does not influence the binding force supplied by the second and third groupings. Similar findings were obtained in inhibition of the 15 system by a pentasaccharide flavazole compound of 3,15 oligosaccharide carrying no galactosyl group as a nonreducing terminal (Robbins and Uchida, 1962b).

The structural similarity of the mannosyl-rhamnose disaccharide obtained from antigens 3, 10 and 3, 15 was again revealed in the inhibition analysis of the 3 system. The evidence that the disaccharides mannosyl-rhamnose from different antigens have similar inhibitory activity for both the 3 and the 15 systems indicates an identity of structure of this disaccharide through the E-group Salmonella antigens.

S. typhimurium, O-antigen 4, 5, 12 contains a galactosyl-mannosyl-rhamnose residue as a major component of its lipopolysaccharide. The mannosyl-rhamnose disaccharide was prepared from the lipopolysaccharide of *S. typhimurium* strain LT2 and tested for inhibitory activity on the anti-3 system. No inhibition was observed with amounts of disaccharide ranging from 0.0025 to 1.5 μmoles. Presumably, the mannosyl-rhamnose from *S. typhimurium* differs from that from E-group bacteria in linkage position or in anomeric configuration.

Determinant 10.—From the chemical structure of the 3, 10 polysaccharide it appeared likely that α-1,6-D-galactosyl-D-mannosyl-rhamnose would be the 10 determinant. Yet, no inhibition was observed by quantities up to 5 μmoles of this trisaccharide with any of several anti-10 sera prepared from different rabbits. None of the oligosaccharides tested inhibited the 10 systems. This suggested either that the 10 determinant involved large areas of the polysaccharide or that some labile radical attached to the α-galactosyl-mannosyl-rhamnose residue was responsible for the specificity of the 10 determinant. It has been estimated that the largest size of polysaccharide determinant unit to which an antibody-combining site may be complementary is a hexa- or heptasaccharide (Kabat, 1960). It has been shown that a trisaccharide segment contributes 75 to 90% of the free energy of binding (Kabat, 1956). Hence, the first possibility was considered less likely than the second. In the recent work of Kotelko *et al.* (1961), antigen 5 was shown to contain an acetyl group whose reactivity is destroyed by alkali. Dr. H. Uetake (personal communication)

suggested to us that a similar structure might be present in the 10 determinant.

A study was made of the effect of alkali treatment on the reactivity of various polysaccharides from group E Salmonella. Only the 3, 10 lipopolysaccharide proved to have alkali-labile activity (Table II). The lipopolysaccharides were treated with 0.01 N NaOH at room temperature, then neutralized with 0.01 N HCl. With 3, 10 polysaccharide and anti-10 serum, there was a significant decrease in complement fixation after 10 minutes of treatment. After 30 and 60 minutes no fixation was observed with antigen quantities that gave the maximum fixation with intact lipopolysaccharide. The optimal quantity of antigen shifted to the extreme region of antigen excess. Lipopolysaccharides 3, 15 and (3), (15), 34 showed no change in their reactivities (Table II). The alkali-treated 3, 10

TABLE II

COMPLEMENT FIXATION WITH ALKALI-TREATED LIPOPOLYSACCHARIDES

The values represent per cent complement fixation. The antisera were used at the dilutions that gave peak fixation with the untreated antigens. The peaks were observed in the same range of antigen quantities as that in control (0.02–0.04 μg). In the case of the alkali-treated lipopolysaccharide 3, 10 and the anti-10 system, a new peak (20–50% fixation) appeared at a high concentration of antigen (0.7–1.4 μg).

Lipopolysaccharide	Time of Treatment with Alkali	Antisera				
		anti-10[a]	anti-10[b]	anti-1, 3, 19	anti-15	anti-34
3, 10	Untreated	80	98	80		
	10 min	53				
	30 min	17		85		
	60 min	<15	65	76		
3, 15	Untreated				92	
	60 min				92	
(3), (15), 34	Untreated					80
	60 min					74

[a] Anti-10 serum absorbed with $A_1(\epsilon^{15})$, *S. senftenberg*, and *S. minnesota*. [b] Anti-10 serum containing cross-reacting antibody for *S. minnesota*.

material still reacts with anti-1, 3, 19 serum with no change in the complement-fixation curve; hence, it appears that the alkali-treated lipopolysaccharide still has enough structure left to exhibit serologic reactivity.

A likely structure for the 10 determinant seemed to be α-galactosyl-mannosyl-rhamnose carrying some alkali-labile radical such as an *O*-acyl group. The lipopolysaccharides from a number of strains were tested for *O*-acyl groups using alkaline hydroxylamine and acidic ferric chloride. The results are presented in Table III. It is clear that the 3, 10 lipopolysaccharide contains *O*-acyl groups. No significant amounts were detected in the 3, 15 and (3), (15), 34 lipopolysaccharides. The content of acyl groups in 3, 10 polysaccharides was 0.47–0.50 μmole acetyl equivalent per mg of the lipopolysaccharide. The molar ratio of acyl group to rhamnose content of the material was 0.54–0.57. This means that only about half the α-galactosyl-mannosyl-rhamnose units carry an acyl group (if only one acyl group exists per unit). The actual value may, however, be higher since it is not known whether the lipopolysaccharide isolation procedure causes deacylation. It has not yet been determined whether there is a difference in acyl content between the 3, 10 lipopolysaccharides of $A_1(\epsilon^{34})$ and of A_1.

TABLE III

ACETYL CONTENT OF LIPOPOLYSACCHARIDES AND RATIO OF ACETYL GROUPS TO RHAMNOSE CONTENT

Rhamnose content of lipopolysaccharide was measured by the method of Dische (1955).

Lipopolysaccharide of	Antigen	μmoles Acetyl per mg Lipopolysaccharide	Acetyl Groups per Rhamnose
A_1	3, 10	0.47–0.50	0.54–0.57
$A_1(\epsilon^{15})$	3, 15	<0.05	<0.05
$A_1(\epsilon^{15}\epsilon^{34})$	(3), (15), 34	<0.05	<0.05

The acyl group was identified as acetyl by comparing on paper chromatograms the hydroxamate derived from polysaccharide 3, 10 with a known acetylhydroxamate.

To test further the nature of the acetylated determinant, oligosaccharides were acetylated by acetic anhydride and used for inhibition analysis. As shown in Figure 7, an acetylated α-galactosyl-mannosyl-rhamnose strongly inhibited the 10 system specifically. Since the hydroxyl groups of hemiacetals and of primary alcohols are acetylated more easily than those of secondary alcohols (Thompson and Wolfrom, 1957), the acetyl groups in the inhibitory oligosaccharide would probably be at carbon 1 of rhamnose and carbon 6 of the galactosyl residue. The most probable structure for the essential part of the 10 determinant appears to be an *O*-acetyl-α-1,6-D-galactosyl-mannosyl-rhamnose structure. As seen in Figure 7, an acetylated β-1,6-galactosyl-mannosyl-rhamnose gave a weak inhibition of the anti-10 system, which may or may not be significant.

Cross-Reaction Between S. anatum and S. minnesota.—As shown in the second column of Table II, alkali-treated lipopolysaccharide 3, 10 still reacted with an anti-10 serum which had been prepared by absorbing anti-A_1 serum with $A_1(\epsilon^{15})$ only. This serum still cross reacts with *S. minnesota* (O-antigen 21, 26: Kauffmann, 1961). This result suggests that determinant 26 is resistant to alkali. As already mentioned, if the acetyl group is attached on the galactosyl residue, only one-half the galactosyl-mannosyl-rhamnose units carry an acetyl group. Hence, anti-A_1 serum may contain some antibody carrying an area complementary to the α-1,6-D-galactosyl-mannosyl . . . configuration; this antibody may be responsible for the cross reaction with *S. minnesota*. We suggest that the 26 determinant group may be an α-1,6-D-galactosyl- . . . structure.

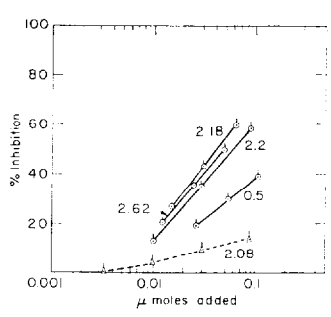

FIG. 7.—Inhibition of system 10 (lipopolysaccharide 3, 10 and anti-10 serum). The symbols used are as follows: ⊙, acetylated α-1, 6-D-galactosyl-1, 4 or 1, 5-D-mannosyl-L-rhamnose; △, acetylated β-1, 6-D-galactosyl-1, 4 or 1, 5-D-mannosyl-L-rhamnose. The numbers on the lines represent the numbers of acetyl groups per trisaccharide unit. Acetyl groups were determined by the method described in the text.

Discussion

In a previous paper (Robbins and Uchida, 1962b) the following picture of the Salmonella O-antigens was presented. The basic unit of the O-specific glycosidic chains would be a galactosyl-mannosyl-rhamnose, which could provide a variety of antigenic specificities by the following modifications: (*a*) changes in position of linkage or in anomeric configuration; (*b*) attachment of some radical—acyl group, dideoxyhexose, or other monosaccharide—to specific positions; (*c*) deletion of one or two of the basic three monosaccharides. The specific structures would be determined genetically through specific biosynthetic processes.

A summary of the chemical nature of the E-group determinants would be as follows. All the Salmonella E-groups contain determinant 3; the corresponding site appears to be the mannosyl-rhamnose structure. Determinant 10 appears to be an acetylated α-1,6-D-galactosyl-1,4(or 1,5)-D-mannosyl-L-rhamnose. Determinant 15 is a β-1,6-D-galactosyl-1,4(or 1,5)-D-mannosyl-L-rhamnose, and determinant 34 is an α-1,4-D-glucosyl-D-galactose site. It should be noted here that Iseki *et al.* (1962) have recently reached a similar conclusion about the chemical nature of determinant 34 by studies on the inhibition by oligosaccharides of the hemagglutination of polysaccharide-coated red cells.

The identification of the reactive site for antigen 3 as the mannosyl-rhamnose structure requires comment. Periodate and methylation studies (Robbins and Uchida, 1962a) indicate that most of these saccharides are internal units. The inhibition data reported here indicate that the galactosyl-mannosyl-rhamnose trisaccharides are as effective inhibitors of the 3 system as mannosyl-rhamnose itself. The most straightforward interpretation is that the specificity of anti-3 antibody is directed against the mannosyl-rhamnose internal units. This interpretation, if correct, limits the validity of Kabat's (1958, 1961) generalization that only the nonreducing terminal groups of a polysaccharide antigen are responsible for the antibody binding sites. Recently Kabat (1962) has modified his views on the possible involvement of internally located sequences as determinant groups as a result of the experiments of Rebers *et al.* (1961) on the S VI anti-S VI system. Although it is conceivable that the inhibition results with the antigen 3 system are fortuitous, the present authors believe it is more likely that internal saccharide units of a polysaccharide may be either antigenic and reactive, or nonantigenic and nonreactive, depending on their nature, configuration, and location in the polysaccharide molecule. There are no theoretical reasons for denying the internal units of polysaccharides a role as major determinant groups of antigenic specificity. Certainly, there are no considerations of protein chemistry that would preclude interactions between such units and the reactive groups of antibody. α-Amylase is an example of a protein that interacts strongly with the internal structure of a polysaccharide. This more general interpretation of antigen determinant groups of polysaccharides does not belittle the importance of nonreducing terminal groups in many antigen-antibody reactions. Thus, the antigen 34 system is an example of the strong determinant role played by nonreducing terminal monosaccharides in Salmonella antigens.

In cases like that of the antigen 15 system, where the determinant group β-1,6-D-galactosyl-mannosyl-rhamnose probably exists both as terminal and as internal units, special methods may be required to establish a possible contribution of the latter units to the antigen-antibody reaction.

In conclusion it may be noted that the changes in polysaccharide structure that are reflected in the serologic specificities of the Salmonella E group are brought about by interactions between the bacterial and phage genomes, presumably at the level of the polysaccharide-synthesizing enzymes. Work is now being carried on to define the nature of the polysaccharide-synthesizing systems and to examine the mechanisms of phage-induced alterations in the polysaccharide structure.

References

Ando, K., and Nakamura, Y. (1951), *Japan. J. Exp. Med. 21*, 149.

Dische, Z. (1955), *Methods Biochem. Analy. 2*, 313.

Fink, K., and Fink, R. M. (1949), *Proc. Soc. Exp. Biol. Med. 70*, 654.

Hestrin, S. (1949), *J. Biol. Chem. 180*, 249.

Iseki, S., Kogure, T., and Yamamoto, S. (1962), *Proc. Japan Acad. 37*, 645.

Kabat, E. A. (1956), *J. Immunol. 77*, 377.

Kabat, E. A. (1958), *in* Chemistry and Biology of Mucopolysaccharides, Wolstenholme, G. E. W., and O'Conner, M., eds., London, Churchill, p. 42.

Kabat, E. A. (1960), *J. Immunol. 84*, 82.

Kabat, E. A. (1961), Kabat and Mayer's Experimental Immunochemistry (2nd ed.), Springfield, Illinois, Thomas, p. 241.

Kabat, E. A. (1962), *Fed. Proc. 21*, 696.

Kauffmann, F. (1942), *Acta Pathol. Microbiol. Scand. 19*, 523.

Kauffmann, F. (1954), Enterobacteriaceae, Copenhagen, Munksgaard, p. 71.

Kauffmann, F. (1961), Die Bakteriologie der Salmonella species, Copenhagen, Munksgaard, pp. 90 and 103.

Kotelko, K., Staub, A. M., and Tinelli, R. (1961), *Ann. Inst. Pasteur 100*, 618.

Lipmann, F., and Tuttle, L. C. (1945), *J. Biol. Chem. 159*, 21.

Rebers, P. A., Hurwitz, E., and Heidelberger (1961), *J. Bacteriol. 82*, 920.

Robbins, P. W., and Uchida, T. (1962a), *Biochemistry 1*, 323.

Robbins, P. W., and Uchida, T. (1962b), *Federation Proc. 21*, 702.

Smith, F., and Montgomery, R. (1956), *Methods Biochem. Analy. 3*, 182.

Staub, A. M. (1960), *Ann. Inst. Pasteur 98*, 814.

Thompson, A., and Wolfrom, M. L. (1957), *in* The Carbohydrates, Pigman, W., ed., New York, Academic, p. 138.

Tinelli, R., and Staub, A. M. (1960), *Bull. Soc. Chim. Biol. 42*, 583.

Uetake, H., Luria, S. E., and Burrous, J. W. (1958), *Virology 5*, 68.

Wasserman, E., and Levine, L. (1961), *J. Immunol. 87*, 290.

Westphal, O., Lüderitz, O., and Bister, F. (1952), *Z. Naturforsch. 7b*, 148.